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PATENT SPECIFICATION

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COMPLETE SPECIFICATION

Method for the Dried Storage of Micro-Organisms

We, COMMONWEATH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION, a body corporate constituted under the Science and Industry Research Act, 1949, of the Parliament of the Commonwealth of Australia, having its head office at 314, Albert Street, East Melbourne, Victoria, Australia, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to an improved method for storage of micro-organisms in a dried viable condition. The expression "micro-organism" is intended to include cellular organisms, viruses, virus-like organisms and components of living cells or tissues for which the recognized biological specificity and potency are dependent on maintaining the integrity of protein or protein-containing molecules or macro-molecules.

It is now well known that many living micro-organisms can be preserved by low temperature drying and retain a significant degree of viability over long periods extending into years. However, it has been appreciated that while an aggregate of dried micro-organisms could be cultivated in a suitable nutrient medium, only a proportion, frequently a low proportion, of the aggregate were in fact viable. This circumstance escaped ready notice as the rapid increase of numbers from a single viable cell could mask the fact that the greater part of the stored material had lost its viability. Such a loss of potency is of great importance as, for example, where a menstrum is applied by volume in the expectation of adding a predetermined count of active material. Any expectation is not fulfilled where any significant proportion of dried micro-organism have died in storage.

The present invention is based upon the discovery that a fundamental cause of death in dried organisms arises from alterations in cell protein brought about by reaction between carbonyl compounds present in cells and amino groups of the cell protein resulting in removal of free amino groups. No discrimination is made between cellular and nuclear protein in the present context.

The above hypothesis is supported by experimental work and statistical analysis of results obtained which show conclusively that addition of carbonyl containing compounds to liquid suspensions of micro-organisms which were subsequently dessicated in accordance with known techniques and sampled periodically to check the viability of the dried organisms, caused marked increase in the death rate with all types of micro-organisms investigated. Tests of this character established that the presence of carbonyl containing substances such as glucose, fructose, galactose, arabinose, ribose, xylose, rhamnose, salts of glucuronic and galacturonic acids, sodium pyruvate, pyruvic aldehyde, and diacetyl all increased the rate of death of micro-organisms under controlled storage conditions. Compounds of relatively low molecular weight such as those with three to five carbon atoms were the most damaging. Compounds with two carbonyl groups per molecule were more destructive than those containing only one such group. It is well known that these and many other carbonyl compounds occur widely in many kinds of living cells and the pentose sugar ribose is known to be a universal constituent of living matter. While all micro-organisms tested have been found sensitive to added carbonyl compounds some species of micro-organisms were found to offer resistance to death from low concentrations of applied pentose. Such resistant forms were found to contain free amino acids within the cell.

Again, the well known Maillard or browning reaction shows that certain reducing sugars and other carbonyl compounds react with protein-amino groups with removal or alteration of free amino groups. Such free group removal or alteration would appear to cause loss of living cell function. Confirmation of the hypothesis is to found in the circumstance that addition of amino acid to liquid suspension of

micro-organisms vastly improved their viability in dry storage. Furthermore, in the prior art it was believed that addition of glucose to liquid suspension of micro-organisms had a favourable influence upon their viability in subsequent dried condition. Substitution of non-reducing sugars for glucose conferred vastly improved viability and analysis of check tests utilising glucose showed that the rate of death of micro-organisms showed a proportional relationship to the initial glucose concentration. It has also been shown that glucose disappears during storage in sealed containers in the substantially dry state and that such destruction of glucose is related to the fall in viability of micro-organisms.

From the foregoing discussion it will be appreciated that sustained viability of micro-organisms in substantially dry stored condition requires means for preventing or substantially inhibiting impairment of cell protein by carbonyl compounds.

Accordingly the present invention consists in a method for obtaining dried micro-organisms which will withstand storage with retention of viability characterised in that one or more substances which inhibit or prevent damage to the micro-organisms resulting from reaction between cellular protein and available carbonyl compounds is incorporated with said organisms.

The invention also includes a method for obtaining dried micro-organisms which will withstand storage with retention of viability comprising the steps of forming a liquid suspension of such organisms and adding thereto substances which inhibit or prevent damage to the micro-organisms normally resulting from reaction between cellular protein and available carbonyl radicals, and evaporating the liquid suspension to secure substantially dry organisms, suitable for storage in sealed containers. In addition, or alternatively to the adding of substances prior to drying, the invention includes the addition of volatile substances after drying, using such volatile substances as are known to inhibit reactions between available carbonyl compounds and protein.

Substances which inhibit or prevent damage to cellular protein from carbonyl groups and which are not themselves deleterious to micro-organisms include non-reducing sugars and polyols including hexitols and penitols and cyclic compounds such as inositol, free amino acids which react with carbonyl radicals in the dry or near dry state, and substances which consume or fix carbonyl compounds. The substances include those known to chemists as carbonyl reagents and include such diverse substances as hydroxylamine, phenyl hydrazine, semicarbazide, pyridinium-aceto-hydrazide chloride (Girard's reagent P) and trimethyl ammonium-aceto-hydrazide chloride (Girard's reagent T). It will be appreciated that some of these substances e.g. hydroxylamine are toxic to many organisms and, therefore, micro-organisms cannot safely be exposed to high concentrations prior to drying. It is a remarkable fact, however, that even low concentrations of the order of 0.01 molar added prior to drying are markedly protective against death of microorganisms stored under substantially dry conditions. The invention, therefore, establishes that substances which are ordinarily toxic under wet conditions may nevertheless be markedly protective under dry conditions. The reverse is true for many carbonyl compounds which are normal constituents of living cells and become lethal only under substantially dry conditions.

It is to be noted that the various protective substances disclosed by the invention are not substances which would be antigenic, that is they are not substances which would elicit th formation of anti-bodies following injection into animals. According to the prior art it was claimed that protective colloids were important in preventing death of dried mocro-organisms and substances such as serum and other colloidal substances were commonly added. Controlled tests have now shown that the non-dialysable components of serum are, in fact, much less protective than the low molecular weight components which diffuse through semi-permeable membranes. It is therefore a feature of the invention that where living organisms are to be dried for use as living vaccines that the organisms may be purified free from unwanted colloidal material and subsequently treated by adding low molecular weight protective substances of one or more of the types previously mentioned and then dried for storage in sealed containers. Preparations so treated avoid the injection of unwanted foreign antigens, a feature which is of special value in human medicine.

As already known from studies of the Maillard reaction it has been known that the reaction is greatly dependent on temperature and the rate increases some 5 to 10 fold for an increase of 10° C. The reaction is decreased by increasing the concentration of hydrogen ions and by decreasing the equilibrium humidity of the system below 60 to 70 per cent. These three factors have been studied with micro-organisms. The rate of death at controlled relative humidity increased greatly about 7 to 10 fold for each 10° C. increase in temperature between 0 and 30° C. Temperature was particularly important in the presence of glucose. For prolonged storage the use of low temperatures will still be advantageous, but stabilization achieved by the invention makes storage at higher temperatures possible. This is a special advantage for distribution of dried vaccines in hot climates when refrigeration was not available.

The expectation that reduced rates of death

would be realized by drying suspensions with increased concentration of hydrogen ions is borne out by experiment with some micro-organisms but it is disadvantageous when the increased acidity leads to hydrolysis of microbial polysaccharide and so generates an increased concentration of lethal carbonyl compounds.

The Maillard reaction becomes important when most of the water has evaporated and this stage is reached during the drying process. In the prior art it was recognized that sensitive organisms could only be dried successfuly at very low temperatures, a procedure which was very slow and costly. The stabilization achieved by the invention makes drying at higher temperatures possible.

It is well known to biologists that the amount of residual water in suspensions of dried micro-organisms affects the retention of viability during storage. There is, however, no agreement regarding the most favourable water contants for survival and some authorities advise that cultures should be as dry as possible. Regulations in U.S.A. provide for water contents not exceeding one per cent. Requirements for very low water contents are most necessary when death following the Maillard reaction is not otherwise inhibited, e.g. in systems containing added glucose or when other naturally occurring carbonyl compounds are free to react with cellular matter. It is a feature of the invention that when Maillard reactions are effectively inhibited that rigorous drying to very low residual water contents is no longer essential. In the presence of stabilizing compounds the optimum water contents for survival are often well above one per cent and may with certain organisms exceed ten per cent. The attainment of the most favorable water contents for survival is best achieved by water vapour equilibration *in vacuo* over solutions maintaining the desired relative humidity. Cultures with their water contents so adjusted may then be sealed in the usual manner either with or without the inclusion of a small porous head or water-vapour permeable capsule containing a saturated solution of a component chosen to maintain the desired humidity indefinitely within the sealed container. The latter procedure would be advantageous for very prolonged storage as water can be generated by chemical reactions of the Maillard type within sealed containers.

Micro-organisms to which the present invention has particular application include:—

Vaccines comprising suspensions of bacteria, rickettsiæ, pleuro-pneumonia like organisms and viruses.

Root nodule bacteria for leguminous plants,

Bacteria used in cheese making

Yeasts

Mammalian spermatoza

As various micro-organisms differ widely in structure and composition and in their resistance to various environmental influences it is not to be expected that a unique set of conditions exists which would be ideal for the stabilization of all micro-organisms. Skilled biologists will appreciate the need for determining which of the substances of the types described in the invention are best suited for the particular micro-organism to be preserved having due regard to the use for which the micro-organisms is required and for any special conditions of storage which might be imposed.

The following examples illustrate the effects of various treatments on some selected micro-organisms. When a culture of *Streptococcus lactis,* a micro-organism used in the manufacture of cheese, was dried in broth containing a one-tenth molar concentration of mannitol the numbers of viable cells was unchanged after 11 months *in vacuo* at 25° C. and 40 per cent relative humidity. When dried in the same broth without added mannitol and stored for the same time under the same conditions survival was reduced to 25 per cent. When dried in the same broth with one tenth molar xylose substituted for the mannitol storage under the same conditions caused rapid death to less than one-millionth of the initial number within 5 days even though 100 per cent of the cells survived the initial drying process.

A strain of *Rhizobium leguminosarum,* an inoculant for clover seeds, when dried in tap water and stored *in vacuo* at 25° C. and 0 per cent relative humidity showed 99.9 per cent death within 10 days but little change within 3 months thereafter. When dried in a one molar solution of sucrose and stored under the same conditions 25 per cent of the cells remained viable after 3 months. A mixture of one tenth molar lysine in one molar sucrose showed similar benefits.

After storage for 8 months survival in material dried in sucrose and stored at 0 per cent relative humidity had fallen to 4 per cent, but remained as high as 25 per cent for similar material stored at 11 per cent relative humidity.

When *Salmonella newport* was dried from a water suspension only 8 per cent of the cells survived the initial drying process. Survival was increased to 30 per cent when these cells were dried in 1.1 molal arabinose and to 50 to 80 per cent when dried in 1.1 molal sucrose or glucose. During storage *in vacuo* at 25° C. the number of survivors fell from 10,000,000,000 per ml. to zero within one week at 43 per cent relative humidity in the presence of arabinose, and at the same relative humidity within 7 weeks in the presence of glucose. When dried from water there were still over 300,000 viable cells after 6 months at 43 per cent relative humidity, most of the death having occurred within the first week of storage. When dried from sucrose solution the organisms surviving for 6 months

greater and equal to some 300,000,000. Storage results at 43 per cent relative humidity were rather similar *in vacuo* and in air. When stored at 0 per cent relative humidity cultures dried in water gave some 3,000,000 survivors after 6 months *in vacuo* but became sterile in about 2 months in air. Cultures dried from arabinose became non-viable within 6 months *in vacuo* and in about 3 months in air. Cultures dried in glucose resulted in about 25 per cent survival after 6 months *in vacuo* and 4—5 per cent after the same period in air. Cultures dried in sucrose gave about 25 per cent survival after 6 months *in vacuo* and also in air. These experiments show that extreme drying, as obtained by storage over phosphorus pentoxide, is not itself sufficient to prevent destruction by pentose sugars and is undesirable when cultures are stored in air. On the other hand any residual moisture accelerates death when reducing sugars are present either in air or *in vacuo*. The non-reducing sugar gives best survival both in the presence and absence of residual moisture and in air or *in vacuo*.

Other experiments have shown that death of all micro-organisms tested was increased by the addition to the suspension to be dried of one tenth molar ribose and that even one thousandth molar concentrations of this sugar significantly increased death of some organisms. Similar low concentrations of other compounds such as diacetyl were also destructive.

When *Salmonella newport* was dried from a simple phosphate buffer solution containing one hundredth molar ribose some 4,000,000,000 cells per ml. survived the initial drying. From an initial 25 thousand million cells per ml. At 43 per cent relative humidity *in vacuo* at 25° C. this number fell to less than 1000 within one week but when one hundredth molar hydroxylamine was also added the lethal effect of the ribose was largely reversed and 10,000,000 cells were viable at this time and 300,000 cells were still surviving after 2 months. When the same cultures were stored at 22 per cent relative humidity protection by small concentrations of carbonyl reagents was even better. In the absence of added carbonyl reagents one hundredths molar ribose caused a reduction to 2,000 per ml. within one month, but after two months there were 100,000,000 living cells when the cultures contained one-hundredth molar hydroxylamine or semicarbazide or Girard P reagent. Substantial protection was also given by Girard T reagent and by sodium borohydride added at the same concentration. The invention therefore demonstrates a 50,000 fold increase in survival by adding only a one-hundredth molar concentration of one of several reagents which are known to combine with carbonyl compounds. The invention, therefore, makes it possible to prevent death

izing substances, the amount of hydroxylamine in the example just cited being one part in some 3,000 parts of suspension to be dried.

When a strain of *Saccharomyces cerevisiæ*, a yeast used in baking, was dried from broth about 30 per cent of the cells survived after 11 months *in vacuo* at 0 per cent relative humidity at 25° C. Under the same conditions of storage the addition of one tenth molar lysine increased survival to 100 per cent and one tenth molar xylose reduced survival to one per cent. The xylose was much more lethal at 43 per cent relative humidity, 99 per cent of the cells then being destroyed in only six days.

When a broth containing staphylococcus bacteriophage race 51, a type of virus, was dried some 40 per cent of the particles survived the initial drying process of 5 hours duration. Addition of one tenth molar lysine to the broth increased survival to 50 per cent whereas addition of one tenth molar ribose reduced it to 1 per cent. During storage *in vacuo* at 25° C. cultures containing the added ribose became devoid of living virus within 2 days at 43 per cent relative humidity and within 4 days at 0 per cent relative humidity. In the broth without supplement the titre of virus fell rapidly to less than 1000 particles per ml. in 3 weeks whereas in broth with added lysine there were over 1,000,000 particles per ml. at this time. At 0 per cent relative humidity death was slower but in unsupplemented broth the viable count had fallen to 8 per cent of the initial in two months whereas in broth with added lysine over 30 per cent of the pre-drying count were still viable after 12 months.

With another virus, the bacteriophage T5 for *Escherichia coli*, drying in broth with or without one tenth molar lysine gave only 4 per cent of virus surviving the initial drying of 5 hours whereas if one tenth molar ribose was added survival at this stage was reduced to one tenth of one per cent. Cultures dried with ribose suffered a further decline of 100,000 fold in 2 days at 43 per cent relative humidity and in 5 weeks at 0 per cent relative humidity. Cultures with added lysine showed no detectable loss in viability in 3 months at 0 per cent relative humidity at 25° C. whereas in the unsupplemented broth there was in this time a further reduction of about 95 per cent of the virus surviving the initial drying.

Although bacteriophage T5 was most stable at 0 per cent relative humidity in broth or broth lysine mixtures a mixture of one-half molar sorbitol and one tenth molar amino acids containing equal amounts of glutamic acid, glycine and lysine was more protective at 43 per cent relative humidity than at lower relative humidities. At 43 per cent relative humidity *in vacuo* at 25° C. there was no significant death in 2 months, as many as 30 per

cent of the pre-drying titre being viable after this time. As the sorbitol amino acid mixture was actually destructive at 0 per cent relative humidity, this example shows the importance of choosing the most favourable relative humidity for the particular micro-organism. Other experiments with this bacteriophage showed inositol to be valuable for increasing survival both during drying and during storage and that glucose, galactose and mannose were all unsatisfactory.

A strain of *Herpes simplex* was dried in a buffered egg allantoic fluid. The virus was more stable at 0 per cent relative humidity than at 43 per cent and after storage for 5 weeks *in vacuo* at 25° C. the titre of virus in material containing one tenth molar added lysine was about 20 times greater than in the control material dried without added lysine.

Other experiments with a strain of human influenza virus have shown that the dried virus was rapidly destroyed by added arabinose and substantially potected by added alanine.

The invention is applicable to the production of vaccines in substantially dry condition for storage. In the production of dried vaccines a fluid suspension of suitable micro-organisms is freed of unwanted colloidal or antigenic substances in known fashion after which there is added to the organisms one or more substances which inhibit or prevent damage to the micro-organisms arising from reaction between cellular protein and available carbonyl compounds, and which further are non-antigenic substances.

The liquid is then evaporated and the substantially dry micro-organisms are sealed in suitable containers. If desired the container atmosphere contains sulphur dioxide for the purpose of added inhibition of destructive reaction between cellular protein and available carbonyl compounds.

Also the preferred carbonyl reagents added to the liquid suspension are selected from the group of substances comprising hydroxylamine, phenylhydrazine, semi-carbazide, pyridinium-aceto-hydrazide chloride and trimethyl ammonium-aceto-hydrazide-chloride and sulphur dioxide.

WHAT WE CLAIM IS:—

1. A method for obtaining dried micro-organisms which will withstand storage with retention of viability characterised in that one or more substances which inhibit or prevent damage to the micro-organisms resulting from reaction between cellular protein and available carbonyl compounds is incorporated with said micro-organisms.

2. A method for obtaining dried micro-organisms which will withstand storage with retention of viability comprising the steps of forming a liquid suspension of such organisms and adding thereto substances which inhibit or prevent damage to the micro-organisms resulting from reaction between cellular protein and available carbonyl compounds, and evaporating the liquid suspension to a substantially dry condition having a water content below that supporting growth of micro-organisms and placing the dried product in containers to be sealed for storage.

3. A method according to Claim 1, in which the one or more substances which inhibit or prevent damage to the micro-organisms are volatile substances added to and sealed with dried micro-organisms in storage containers.

4. A method according to Claim 2, in which sulphur dioxide is added to the dried product and sealed therewith in storage containers.

5. A method according to any one of the preceding claims wherein substances which inhibit or prevent damage to the micro-organisms are non-reducing sugars and polyols, cyclic compounds such as inositol, free amino acids which are reactive with carbonyl compounds in the dry or near dry state and carbonyl reagents.

6. A method according to Claim 5 in which the carbonyl reagents are hydroxylamine, phenyl hydrazine, semi-carbazide, pyridinium-aceto-hydrazide chloride and trimethyl ammonium-aceto-hydrazide-chloride and sulphur dioxide.

7. A method for obtaining vaccines in substantially dry condition for storage comprising the steps of removing unwanted colloidal or antigenic substances from a fluid suspension of micro-organisms and adding one or more non-antigenic substances which inhibit or prevent damage to the micro-organisms resulting from reaction between cellular protein and available carbonyl compounds and evaporating the liquid to secure substantially dry organisms and storing the dried product in sealed containers.

8. A method for obtaining dried micro-organisms which will withstand storage with retention of viability substantially as described with reference to the examples.

9. Dried micro-organisms whenever obtained in accordance with the method of any one of the preceding claims.

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